Vascular Functions of NADPH Oxidases

Ralf P. Brandes

NADPH oxidases belong to a group of enzymes that generate reactive oxygen species (ROS) by electron transfer from NADPH to molecular oxygen. The product of this reaction is the superoxide anion ($O_2^-$), which undergoes secondary reactions. $O_2^-$ inactivates NO to yield peroxynitrite and spontaneously or under catalysis of superoxide dismutases reacts to hydrogen peroxide. NADPH oxidases, therefore, limit vascular NO availability and facilitate reactions involving ROS. It is now well understood that endothelial dysfunction is largely a consequence of NADPH oxidase activation, as well as of complex secondary reactions that involve different types of ROS. Peroxynitrite oxidizes the NO synthase cofactor tetrahydrobiopterin and stimulates kinases, which both lead to uncoupling of the endothelial NO synthase. NO and $O_2^-$ may also affect gene expression, and, in particular, NADPH oxidase–derived hydrogen peroxide has been shown to be important in this respect. In addition to the stress-mediated ROS-stimulated gene expression, hydrogen peroxide transiently or irreversibly oxidizes biological materials and, therefore, has complex effects on signaling that are still incompletely elucidated.

NADPH Oxidases From Past to Present

Although the initial work on NADPH oxidases focused on its role for gene expression and endothelial dysfunction at large, the research of recent years aimed at the identification of specific NADPH oxidase–dependent signaling pathways, the understanding of the role of the enzymes in individual disease entities, the identification of NADPH oxidase inhibitors, and finally the demonstration of a relevance of NADPH oxidases in human cardiovascular diseases. Indeed, it is now known that patients with chronic granulomatous disease, which results from loss-of-function mutations of the NADPH oxidase, have an increased vascular NO availability and reduced vascular levels of footprint markers of ROS formation. This important observation provided a direct link between NADPH oxidase and endothelial function in humans. Chronic granulomatous disease, however, is too rare and too severe to study whether the advantage in endothelial function also translates into a reduced cardiovascular disease rate. Studies on polymorphisms of NADPH oxidase genes, however, failed to draw a clear picture. Thus, most data in support of a relevance of NADPH oxidases for human cardiovascular disease are based on indirect treatment studies. Several highly effective cardiovascular drugs, such as statins, peroxisome proliferator-activated receptor-γ agonists, angiotensin-converting enzyme inhibitors, and angiotensin II type 1 (AT$_1$) receptor antagonists prevent the activation or induction of NADPH oxidases and reduce the oxidative burden in patients. The possible conclusion that NADPH oxidases are excellent cardiovascular drug targets might, however, still be premature, in particular, in light of the failures of large prospective trials testing antioxidants. These studies demonstrated that, although effective in the acute setting, antioxidant treatment faces rapid development of resistance resulting from the readjustment of the antioxidative balance. Indeed, the concept of ROS toxicity as a causal factor for chronic disease processes has been eroded by the observation that endogenously produced ROS serve important functions in compartmentalized physiological signaling processes and that sensitive signaling networks actively control the redox balance of the cell. Therefore, it remains to be determined whether isoform-selective NADPH oxidase inhibition will circumvent the counterregulatory reactions observed with antioxidants.

Substantial work has been devoted to characterize isoform-specific signaling pathways and effects mediated by the individual NADPH oxidases. In fact, this class of enzymes consists of 7 members, which are named by the large, catalytically active Nox protein (Nox1 through 5 and Duox 1 and 2). Of these, Nox1, 2, 4, and 5 are expressed in the vessel wall with different cell-specific and pathology-associated expression patterns. Several different transgenic mice for the different oxidases were generated, and these were instrumental in the understanding of the functions of individual NADPH oxidases and also instructive regarding limitations of the system. For example, Nox knockout mice presented with an attenuated hypertensive response to angiotensin II, suggesting a great importance of redox regulation for high blood pressure development. However, when models of lifelong hypertension and Nox deficiency were combined, for example, by crossing Nox1 knockout mice with mice carrying a transgene for a hepatic expression of the active human renin (TTRhRen mice), hypertension was not affected by knockout of the NADPH oxidase. The fact that the expression of proinflammatory adhesion molecules, such as vascular cell adhesion molecule 1, and the activation of redox-sensitive kinases, such as the jun N-terminal kinase, however, were lower in TTRhRen/Nox1$^-/^-$ mice suggests that some undefined counterregulatory system in this setting mediates the loss of the antihypertensive effect.
Not only for these types of studies are knockout animals essential, they are also of particular importance because selective NADPH oxidase inhibitors are still not yet available. The most frequently used inhibitory compound, apocynin, was observed to act as an antioxidant at higher concentrations. Thus, in studies using concentrations of apocynin >100 μmol/L, beneficial effects of the compound were generally documented. Unfortunately, these were not linked to the antioxidative properties of apocynin but lead to a rather unwarranted association of NADPH oxidases to basically every cardiovascular disease.

**Recent Directions of NADPH Oxidase Research**

This lack of pharmacological inhibitors weighs heavily on the field because it limits studies in patients and because inhibitors derived from molecular biology, such as small interfering RNA, also have limitations. It is not just that in vivo studies with tools like small interfering RNA are still only possible in a few centers; such experiments are prone to artifacts derived from the transfection technique, the time required to yield effective suppression of the gene of interest, and potential unspecific actions. Nevertheless, a relatively large number of excellent studies employing such tools have been published recently, shifting the focus on NADPH oxidases to a molecular understanding of their role in central and renal mechanisms of hypertension, the metabolic syndrome, and secondary cardiovascular diseases, such as myocardial hypertrophy or interstitial fibrosis. It might be important to mention that it is far from clear whether ROS are the source, consequence, or both of hypertension. Although it is well accepted that ROS limit NO availability, this process does not necessarily result in hypertension. Moreover, hypertension itself increases the ROS formation of NADPH oxidases: circumferential wall stress activates the integrin linked kinase 1, which, through the guanine nucleotide exchange factor β-Pix catalyzes Rac-1 GDP-GTP exchange and subsequent NADPH oxidase activation. The consequence of this is an attenuation of the endothelium-dependent relaxation and, thus, potentially an increase in peripheral resistance, which, at least in theory, should further promote hypertension development.

**NADPH Oxidases and Hypertension Involving the Central Nervous System**

The central nervous system plays a major role in the control of blood pressure. Through the sympathetic tone, the central nervous system regulates heart rate and cardiac output, as well as peripheral resistance and venous tone. It directly or indirectly controls hormones involved in salt and water retention, like renin or vasopressin, and through the regulation of thirst and salt appetite affects volume intake. Down-regulation of NADPH oxidases directly in the brain documented a complex role of the oxidases in these systems: small interfering RNA directed against Nox2, for example, prevented the angiotensin II–induced dypsogenic effect, and downregulation of Nox2 and Nox4 in the brain prevented the hypertensive effect of angiotensin II. A cross-talk between NADPH oxidase– and mitochondria-dependent ROS formation has been discussed for some time. Apparently, this interaction is also important in the brain: mitochondrial uncoupling is observed in response to angiotensin II in the brain of normal rats, and, importantly, in spontaneously hypertensive rats this uncoupling even occurs under basal conditions. The complex interplay of angiotensin II–induced ROS formation and cerebral function is also illustrated by the impaired cognitive function of human renin and human angiotensinogen gene chimeric transgenic (hRn/hANG-Tg) mice. In these animals, high expression of Nox4 and p47phox was observed. Blockade of the AT1 receptor and scavenging of O2− prevented the cognitive impairment. It is, however, unknown whether the mechanism underlying this phenomenon is ROS-mediated neuronal toxicity or ROS-induced vasoconstriction, resulting in cerebral hypoperfusion and hypoxia. Other recently addressed aspects relate to the role of NADPH oxidases in cerebral vessels, where the enzyme appears to play a role for the development of vasospasms. Application of blood to the subarachnoidal space in mice increased the expression and activity of the Nox2-containing NADPH oxidase in cerebral vessels by a pathway involving increased formation of tumor necrosis factor-α. Blockade of NADPH oxidase activation by inhibition of Rac1 or tumor necrosis factor-α neutralizing antibodies prevented vasospasm development in response to blood application and reduced the blood-induced brain damage.

**NADPH Oxidases in Renal Hypertension**

The expression of NADPH oxidases has been documented in almost every cell of the kidney, and NADPH oxidases have been linked to renal fibrosis and disturbed function of the proximal tubule. A role for NADPH oxidases has been particularly suggested for the tubuloglomerular feedback: Nox2 and Nox4 are expressed in the macular densa, and stimulation of this region with NaCl induces an Nox2-dependent ROS production. It is plausible that, through this mechanism, the tone of the afferent glomerular artery increases, which reduces glomerular perfusion. Moreover, NADPH oxidase–derived ROS decrease the renal endothelial NO synthase expression, and this mechanism was observed to contribute to salt retention in angiotensin II–induced hypertension.

In cells of the proximal tubule, the NADPH oxidase components Nox2, Nox4, and Rac1 are expressed and even further induced by hypertension. Interestingly, a role for lipid rafts in controlling NADPH oxidase activity in the proximal tubule has been suggested through an action on the oxidase activator Rac1: under nonstimulated conditions, membrane-bound Rac1 appears to be associated with lipid rafts. Destruction of rafts by cholesterol depletion activates the ROS formation from Nox2 and Nox4 NADPH oxidases in human proximal tubule cells. Importantly, it was observed that dopamine D1 receptor agonists also release Rac1 from lipid rafts and thereby induce an agonist-stimulated ROS production in the kidney. However, it should be mentioned that overexpression studies failed to link Rac1 with Nox4 activation. Under the latter condition, Nox4 is, however, also predominantly observed in the endoplasmic reticulum, and it is currently unknown whether interacting proteins as are present in smooth muscle cells associate Nox4 with lipid rafts in the kidney. Several observations link NADPH oxidases with glomerular sclerosis and renal fibrosis. In the kidney of rats with...
NADPH Oxidases and Components of the Renin-Angiotensin-Aldosterone System Beyond the AT₁ Receptor

The AT₁ receptor is considered to be the main stimulus for vascular NADPH oxidases, whereas angiotensin II type 2 receptors may even block NADPH oxidase activation.38 Also, the MAS receptor, which is activated by angiotensin (1-7)
appears to limit NADPH oxidase expression and activation. MAS knockout mice exhibited an increased vascular expression of Nox2 and higher vascular ROS levels than control mice. Importantly, these effects were accompanied by endothelial dysfunction and hypertension. Similar to what has been suggested for the angiotensin II type 2 receptor, MAS receptors activate the phosphatase SHP-2, which subsequently dephosphorylates c-src, a signaling tyrosine kinase that acts as an important mediator of the AT1 receptor.

Several recent publications established a role of NADPH oxidases for the effects mediated by aldosterone and the mineralocorticoid receptor (MR). In TG(mRen2)27(Ren2) hypertensive rats, MR blockade reduced NADPH oxidase activity, footprint markers of oxidative stress, and vascular injury. Similarly, treatment of rats with renovascular hypertension with the MR receptor blocker eplerenone reduced the incidence of cerebral aneurysms, which was accompanied by an attenuated vascular staining for the ROS footprint marker nitrotyrosine, and reduced expressions of Nox4 and Rac1, as well as attenuated ROS formation. Tissue-specific expression of the MR in mice demonstrated that the oxidative stress induced by angiotensin II is in part mediated by the action of mineralocorticoids. Interestingly, cardiac fibrosis in response to aldosterone is lost in mice with a myeloid-specific knockout of the MR. These animals also exhibit a reduced cardiac Nox2 expression, which is suggestive for a potential role of macrophages and Nox2 in the action of aldosterone. Whether the attenuation of cardiac Nox2 should, however, only be seen as a marker for macrophage accumulation is unclear. Nevertheless, Nox2 appears to play a central role in the heart: after permanent coronary ligation, Nox2 knockout mice develop smaller infarcts, less dilatation, and, subsequently, less hypertrophy and fibrosis as compared to wild-type mice.

The molecular ROS-dependent signaling mechanisms activated by the MR are still unclear. The apoptosis signal-regulating kinase 1 was considered an important redox-sensor because it measures the level of reduced thioredoxin and increases the phosphorylation of redox-stimulated kinases, such as jun N-terminal kinase. Genetic deletion of apoptosis signal-regulating kinase 1 attenuates the MR-stimulated cardiac fibrosis and ROS formation. Because apoptosis signal-regulating kinase 1, however, is also required for the MR-stimulated induction of Nox2, its role as redox sensor, at least for the MR signaling, is still somewhat uncertain. This important interaction is also demonstrated by studies on thioredoxin 2–overexpressing mice: these animals exhibit a reduced ROS formation and NADPH oxidase expression in response to angiotensin II. Importantly, thioredoxin 2 is located in mitochondria, suggesting a profound cross-talk between mitochondria and NADPH oxidases. The situation is further complicated by the fact that the expression of thioredoxin is also redox sensitive: for example, a coculture of vascular smooth muscle cells with endothelial cells reduces the ROS formation in the latter cell type by an induction of thioredoxin. A complex mixture of endothelial autocoids involving NO, cyclooxygenase 2 products, hydroxyeicosatetraenoic acids, and NADPH oxidase–derived ROS appears to contribute to this effect.

Collectively, these observations suggest that a substantial part of the vascular but also cardiac effects of angiotensin II is a consequence of indirect actions involving the MR-dependent induction and activation of NADPH oxidases. Similar observations, although largely based on pharmacological data, have been made for serotonin and the adrenoceptors: inhibition of the 5-HT(2B) receptor for serotonin reduced the ROS production and cardiac hypertrophy in response to angiotensin II. α2-Adrenoceptor agonists also enhance the NADPH oxidase activity and potentiate the angiotensin II–induced ROS formation and RhoA activation of resistance vessels in the rat kidney.

Conclusions
Whereas the initial work on vascular NADPH oxidases mainly demonstrated an importance of the system for angiotensin II–induced vascular dysfunction, recent studies provided detailed mechanisms regarding how isoforms of the oxidase in the brain and the kidney could contribute to hypertension. They also illustrate that not only angiotensin II but also mineralocorticoids mediate important aspects of their signaling through NADPH oxidases. Finally, more and more systems are discovered that endogenously limit NADPH oxidase activation and expression to fine-tune the redox-signaling network in the vasculature.

Sources of Funding
This study was supported by grants from the Deutsche Forschungsgemeinschaft (SFB815/TP1 and SFB834/TPA2) and the Excellence Cluster Cardio-Pulmonary System.

Disclosures
None.

References


Vascular Functions of NADPH Oxidases
Ralf P. Brandes

_Hypertension_. 2010;56:17-21; originally published online May 17, 2010;
doi: 10.1161/HYPERTENSIONAHA.108.120295

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/56/1/17

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/